Chapter 6

Solvent–Enzyme–Polymer Interactions in the Molecular-Weight Control of Poly(*m*-cresol) Synthesized in Nonaqueous Media

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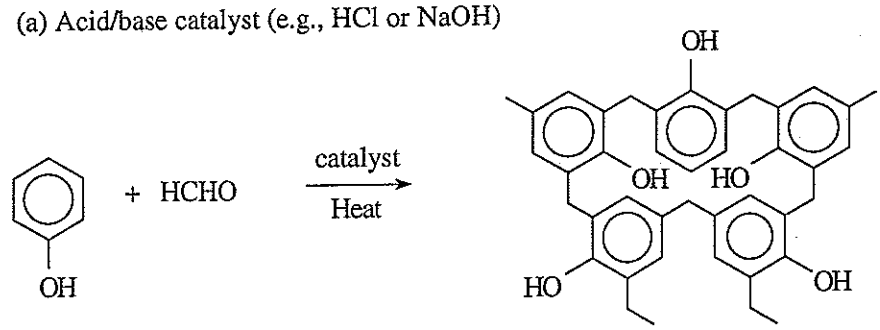
Poly(*m*-cresol) was synthesized by horseradish peroxidase-catalyzed reactions in ethanol/buffer mixtures. Polymer molecular weight was controlled by manipulating the solvent composition. The effect of solvent composition on enzyme activity and polymer solubility was studied to understand the factors affecting polymer molecular weight. Enzyme kinetics revealed the effect of solvent on enzyme activity and substrate partitioning between bulk solvent and the enzyme. Thermal and spectroscopic characteristics of the polymer are discussed.

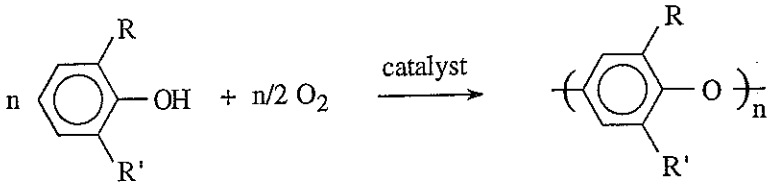
Polyphenols have long been used as engineering materials and specialty polymers. Commercially available phenolic resins such as novolacs and resoles are primarily used as adhesives and laminates. They are prepared in industry by condensing different proportions of phenol and formaldehyde in the presence of an acid or a base catalyst [*1*]. However, polyphenol production by alternate processes is desirable due to the toxicity of formaldehyde. Inorganic catalysts can be used to polymerize phenol or 2,6-derivatives of phenol to make poly(phenylene oxides) in the presence of molecular oxygen [*2*]. Likewise, biological catalysts such as polyphenol oxidases can be used to polymerize phenol and its derivatives or aromatic amines in the presence of hydrogen peroxide or molecular oxygen. The advantages offered by enzymatic processes over chemical processes have been reviewed [*3-8*]. Additionally, polyphenols prepared by enzymatic processes have extensive conjugation in their structure leading to electrical conductivity under doped conditions and nonlinear optical behavior (Figure 1). As a result, these polymers are expected to find wider applications in the fields of electronics and photonics. Although ferric chloride is capable of coupling phenolic units on the aromatic rings leading to conjugated structures, the resultant product is at best dimers or trimers [*unpublished results and 9*].

Peroxidase-catalyzed oxidation of phenols in aqueous media has been known for over 50 years [*10*]. However, a number of substituted phenolic monomers or aromatic amines have very limited solubility in water. Hence, since the advent of nonaqueous enzymology, phenol polymerization in organic solvents by enzyme-mediated reactions has been well studied to produce different types of higher molecular weight polymers [*3-8*]. Among a number of polyphenol oxidases, the enzyme extracted from the roots of horseradish is widely used to catalyze the reaction because of its

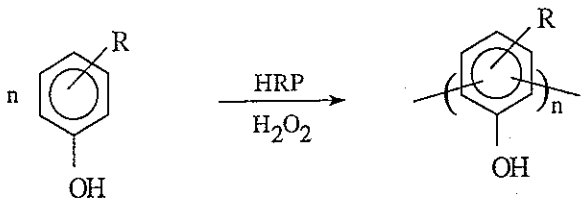
(a) Acid/base catalyst (e.g., HCl or NaOH)



(b) Inorganic catalyst (e.g., copper halide + aliphatic amine)



(c) Biocatalyst (e.g., peroxidases, laccase or tyrosinase)



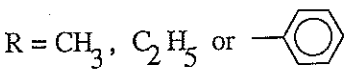


Figure 1: Structures of phenolic polymers synthesized by chemical and enzymatic methods.

availability in pure form at relatively low cost, specificity and activity toward a number of monomers. Horseradish peroxidase (HRP) is active in a number of organic solvents and their mixtures with water [*3-5,8*]. Phenol, derivatives of phenol and aromatic amines have been polymerized and polymers characterized with the help of a variety of spectroscopic techniques [*4*]. Phenol polymerization can be carried out in bulk solvents, at the oil-water interface of reversed micelles and at the air-water interface of Langmuir troughs [*3,4,7,11*]. The polymers exhibit molecular weights ranging from a few hundred to a few tens of thousand depending on the type of monomer and the reaction medium. Significant molecular association of phenolic polymers occurs in solutions of DMF or mixtures of DMF and methanol. This can lead to an overestimation of molecular weights as determined by gel permeation chromatography [*5*]. Inorganic salts such as LiBr, at sufficiently high concentrations, help dissociate the polymer aggregates.

We have reported earlier on the ability to control the molecular weight and polydispersity of polymers from *p*-cresol, *p*-ethylphenol and *m*-cresol synthesized by peroxidase-catalyzed reactions in reversed micelles and mixtures of DMF or ethanol with water [*5,8*]. The ability to control polymer molecular weight and polydispersity is desirable since different applications may require different polymer properties that are dependent on molecular weight. One objective of the work has been to produce low molecular weight oligomers and to analyze their application potential in the photoresist industry. Higher molecular weight materials are desirable for applications as abrasives and foundry materials such as machine housings, automotive transmissions and cylinder heads. Polymer processability is another important factor in a number of applications. The hydroxyl groups on the polyphenols prepared by enzymatic methods are amenable to chemical modifications for melt casting, to render them soluble in alkanes for solvent casting or to enhance their UV absorbing characteristics.

We discuss some of our recent results on polymer characteristics and solvent-enzyme-polymer interactions that lead to a molecular level understanding of the phenomena involved in the control of polymer molecular weight.

Experimental

Phenolic monomers and solvents were purchased from Aldrich Chemical Company (Milwaukee, WI). Horseradish peroxidase (Type II) was purchased from Sigma Chemical Company (St. Louis, MO). All chemicals were of highest purity available, and were used as received.

All synthetic and kinetic reactions were carried out at room temperature. A reaction mixture for polymer synthesis was prepared by dissolving 0.1 to 0.2 M monomer in a mixture of DMF or ethanol and buffer. An aliquot of enzyme solution, prepared by dissolving HRP in N-[2-hydroxyethyl]piperazine-N'[2-ethanesulfonic acid] (HEPES) buffer at a known concentration, was added to the monomer solution such that the final enzyme concentration was 0.1 mg/mL. The reaction was initiated by dropwise addition of H_2O_2. The total amount of H_2O_2 added was 30% in excess of the stoichiometric amount relative to monomer. Reactions were continued for 24 hours. For molecular weight analysis, 50 μL of the reaction mixture was diluted with 3 mL of 1% LiBr/DMF solution. The diluted solution, 50 - 100 μL, was injected onto a gel permeation chromatography (GPC) column. Details of the GPC column and conditions for molecular weight analysis were discussed earlier [*5*]. Polystyrene was used as an external standard.

Experiments to determine the reaction kinetics were carried out with *m*-cresol and HRP in ethanol/HEPES buffer mixtures of different compositions. The enzyme concentration was reduced to 99 nM (4 μg/mL) in order to obtain slower and measurable reaction rates. *m*-Cresol concentration was varied between 10 to 50 mM and 5 mM H_2O_2 was used to initiate the reaction. 100 μL samples were taken at a

predetermined time, depending on the reaction rate, and diluted with 2.4 mL acetonitrile. Molecular weight analysis was done by GPC with 5-10 μL aliquots of this sample. A 3.9 x 150 mm NOVA-PAK C18 reverse phase column (Waters, Milford, MA) was used to estimate the monomer concentration with the help of a UV detector calibrated at 280 nm. A 56/44 (v/v) acetonitrile/water mixture was used as the eluent at a flow rate of 1 mL/min. Each reaction and injection was run in duplicate. Kinetics were analyzed based on the assumption that the reaction followed pseudo-single substrate kinetics in the presence of excess hydrogen peroxide. However, the concentration of hydrogen peroxide was low enough not to inhibit the enzyme.

Reactions in reversed micellar solutions were carried out as described elsewhere [7]. Polymer at the end of reaction was recovered and purified before analyzing molecular weight. Monomer conversion in the supernatant was chromatographically determined. Thermal analysis on the polymers was carried out in a nitrogen atmosphere at a heating rate of 10°C/min.

Results and discussion

The reaction scheme for HRP-catalyzed phenol polymerization is illustrated in Figure 1. ^{13}C-NMR results from earlier studies showed that the monomer units in poly(*p*-ethylphenol) are primarily linked at ortho positions as the para position is occupied by the ethyl group. As a result, the polymer is not expected to be cross-linked. On the other hand, *m*-cresol has three positions for bond formation on the phenylene ring, and the resultant polymer is expected to be highly cross-linked. Thermal characteristics of the polymers support this notion (Figure 2). Differential scanning calorimetry (DSC) thermograms for poly(*p*-ethylphenol) and poly(*m*-cresol) show that the latter is thermally stable while the former exhibits a large exotherm at 116°C, an indication of thermal cross-linking in poly(*p*-ethylphenol) [5]. Thermogravimetric analysis (TGA) showed that the polymers are thermally stable with about 10% weight loss at 300°C in a nitrogen atmosphere.

Details of poly(*p*-ethylphenol) synthesis and polymer molecular weight and polydispersity control in DMF/water systems have been reported earlier [5]. Results for poly(*m*-cresol) synthesized in ethanol/water system are presented here. Polymers of *p*-cresol, *p*-ethylphenol, *p*-isopropylphenol and *p*-butylphenol could also be prepared in ethanol/water mixtures, and the polymers showed a maximum molecular weight of about 2,500. In comparison, poly(*m*-cresol) could be prepared up to a molecular weight of 25,000. Figure 3 shows the effect of ethanol content in the reaction medium on poly(*m*-cresol) molecular weight. The data in Figure 3a reflect the molecular weight of the polymer obtained from 0.2 M monomer. Polydispersity in all cases was greater than two. Although the polymer produced in 100% buffer exhibited a molecular weight of ca. 3,000, the polymer yield was extremely poor. The relatively higher molecular weight of poly(*m*-cresol) compared to para-substituted polyphenols may be attributed to cross-linking in poly(*m*-cresol). A molecular weight of ca. 7,000 was obtained in 20% ethanol/water mixture wherein the polymer yield was over 90%. Earlier we reported molecular weights as high as 25,000 for poly(*m*-cresol) at lower monomer concentrations [8]. Lower monomer concentration at a constant enzyme concentration means lower hydrogen peroxide-to-enzyme molar ratio. This leads to minimal enzyme inhibition, higher monomer conversion and polymer yield and higher molecular weight. Although there is no significant difference in the molecular weight of poly(*m*-cresol) for ethanol content in the reaction medium in the range of 10 to 40%, poly(*m*-cresol) molecular weight exhibited an overall bell-shaped dependence on ethanol content. This 10-40% ethanol range corresponds well with that for which there is a good-to-significant (50-95%) monomer conversion (Figure 3b). It is therefore logical to attribute this phenomenon to molecular interaction of solvent with enzyme, monomer

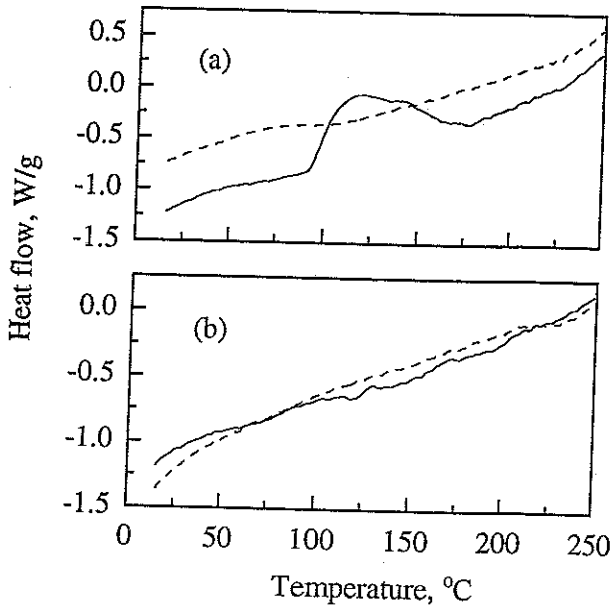


Figure 2: DSC thermograms of (a) poly(*p*-ethylphenol) and (b) poly(*m*-cresol). Solid and dashed lines represent first and second heats, respectively.

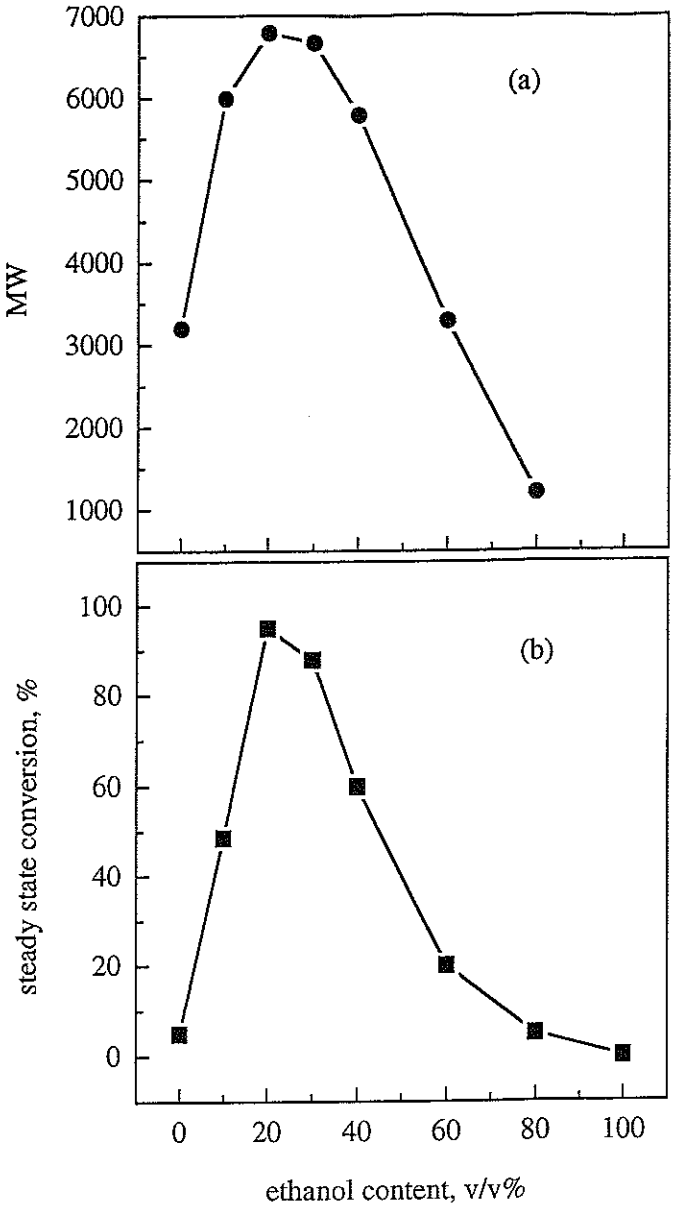


Figure 3: Effect of ethanol content in the reaction medium on (a) poly(*m*-cresol) molecular weight and (b) monomer conversion.

and polymer. While the monomer and polymer are expected to have enhanced solubility at higher ethanol contents, the presence of excess solvent may be detrimental to enzyme activity as shown in a number of solvent-enzyme systems [*12*]. Therefore, there exists a balance between the solubility of growing polymer chains that leads to enhanced molecular weight and the enzyme activity that is responsible for the monomer conversion. We examined these different molecular interactions that are responsible for the observed poly(*m*-cresol) molecular weight profile in ethanol-water reaction medium by measuring enzyme activity, by studying the enzyme structure and by estimating the polymer solubility.

Figure 4 illustrates the idealized profiles of enzyme activity and polymer solubility as a function of solvent composition expected from the estimation of solvent-enzyme-polymer interactions that can be experimentally quantified. From the knowledge of solubility parameters for the solvent mixture and the polymer, the solvent-polymer interaction parameter, $\chi_{poly\text{-}solv}$, can be estimated from Flory-Huggins theory. Polymer chains essentially cease to grow after they precipitate out of solution, and keeping the chains solubilized enhances molecular weight. Since ethanol aids polymer solubility, higher molecular weights can be expected at a higher ethanol content in the reaction mixture. However, the enzyme activity is strongly dependent on ethanol content. As the proportion of ethanol increases in the reaction mixture, enzyme activity goes through a maximum. Analogous to dioxane/water and DMF/water systems, the enzyme is completely deactivated at high concentrations of ethanol. However, the enzyme does not appear to be irreversibly deactivated in 100% ethanol. While the enzyme exhibits no activity in 100% ethanol, gradual addition of buffer (to bring ethanol content from 100% to 20%) with all reactants in place leads to near complete restoration of enzyme activity. This observation suggests that the lack of enzyme activity in ethanol may not be completely due to perturbations caused in enzyme structure. Rather, a combined effect of solvent-monomer-polymer-enzyme interactions as a function of solvent composition is reflected in the observed polymer molecular weight profile. Solvent effect on enzyme activity is studied by estimating kinetic constants and by probing the enzyme structure by spectroscopic methods.

Figure 5 shows the Lineweaver-Burk plots for HRP-catalyzed oxidation of *m*-cresol in a reaction mixture where ethanol content varied from 0% (i.e., all buffer) to 80%. The enzyme was essentially insoluble and inactive in reaction media containing 80 to 100% ethanol. Table I lists the kinetic constants obtained from the plots. The enzyme exhibited most activity (i.e., number of moles of monomer consumed per unit time) in a reaction medium containing 20% ethanol. The Michaelis constant, K_M, decreased continuously as the ethanol concentration increased, which is indicative of the decreasing affinity of the enzyme for the substrate. The apparent loss of enzyme activity may be attributed to the loss of enzyme affinity for the substrate. However, the increasingly weak binding of the monomer to the enzyme may be due to a number of reasons including one or both of the following. (1) The enzyme structure is perturbed so that either the access of substrate to enzyme active site is restricted or the substrate binding is weak; (2) the substrate partitioning between the solvent and the enzyme active site is significantly affected. Ryu and Dordick [*13*] recognized in their study the role of organic solvents on peroxidase structure and function and reported that monomer-solvent interactions dictate the monomer partitioning behavior between solvent and the enzyme active site. As a result, at high ethanol contents, *m*-cresol is likely to be overwhelmingly partitioned to the solvent leading to nonavailability of the substrate in the enzyme active site. We are currently probing the enzyme structure as a function of solvent composition using circular dichroism (CD) spectropolarimetry, fluorescence spectroscopy and room temperature electron spin resonance (ESR) spectroscopy. Preliminary results from CD and UV studies indicate that the enzyme secondary structure and heme environment are invariant with solvent composition. For

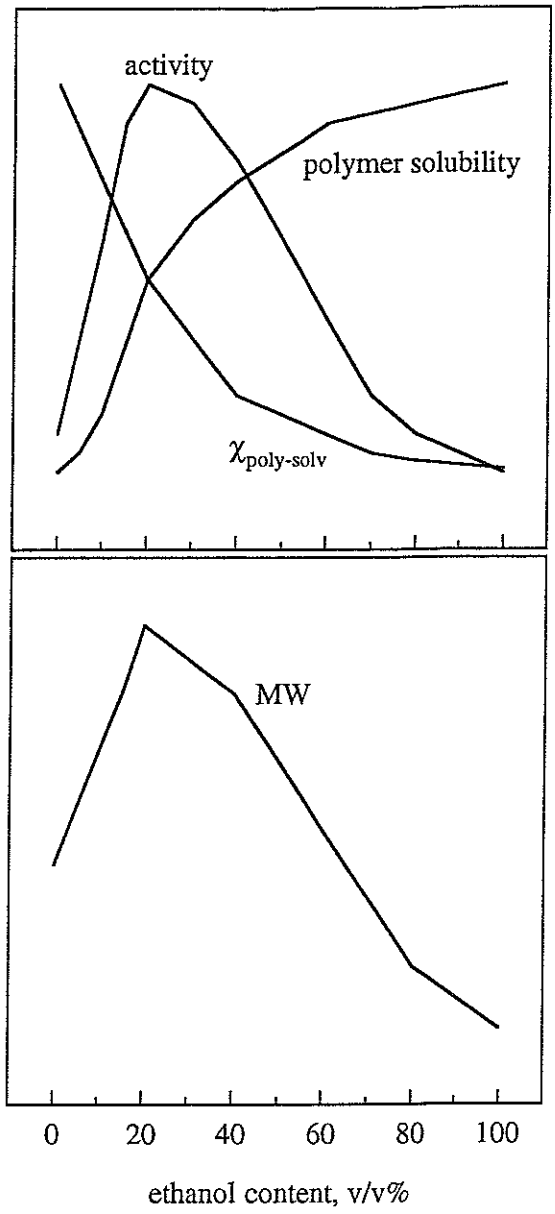
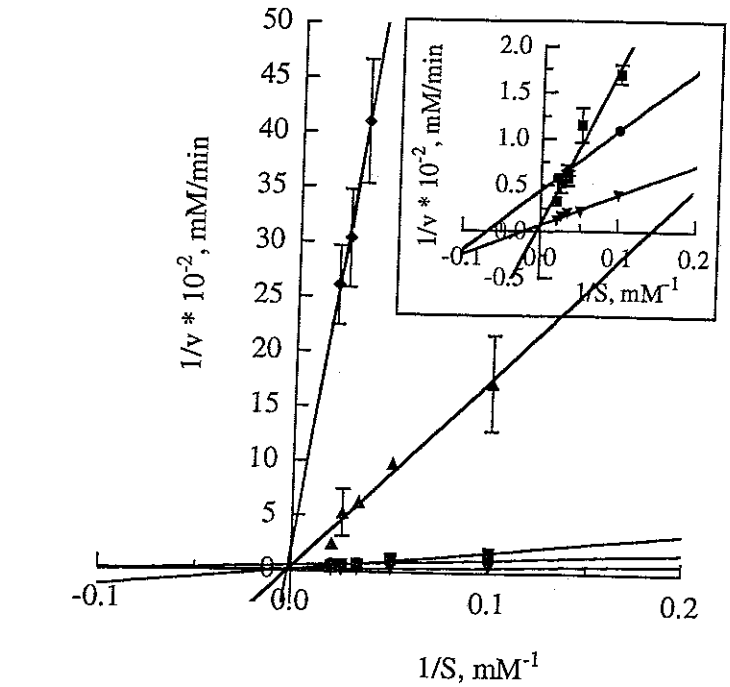


Figure 4: Idealized profiles expected from the estimation of solvent-enzyme-polymer interactions.



(● 0% , ▼ 20%, ■ 40%, ▲ 60% and ◆ 80% ethanol)

Figure 5: Lineweaver-Burk plots for HRP-catalyzed oxidation of *m*-cresol in a reaction mixture with varying ethanol concentration. (Inset: Expanded region for lower ethanol concentration)

ESR measurements, the enzyme protoheme group is spin labeled [*14*]. This labeling allows us to follow the enzyme active site dynamics at room temperature, which are truly reflective of the kinetic behavior of HRP.

Table I. Kinetic data for HRP-catalyzed oxidation of *m*-cresol in ethanol-water mixtures

Ethanol (% v/v)	V_{max} *(mM/min)*	K_M *(mM)*	k_{cat}/K_M $(min*\mu g/mL)^{-1}$
0	221	14	3.9
20	1293	41	7.9
40	1111	187	1.5
60	292	503	0.15
80	47	548	0.02
100	0	-	-

In order to obtain lower molecular weights, *m*-cresol was polymerized in 100% buffer. As seen in Figure 3, the polymer thus prepared exhibited a molecular weight of about 3,000 but the polymer yield was poor. However, this number could be lowered to 1,400 and the monomer conversion could be enhanced to 40% by reducing hydrogen peroxide to enzyme molar ratio. Further, polymer chains could be precipitated as they formed by the addition of small amounts of salt, and the polymer could be recovered by filtration. Monomer conversion could also be enhanced by pulsed addition of the enzyme. A process was developed to produce polymer with control of molecular weight and to enhance monomer conversion (Figure 6). The ethanol content in the feed and the residence time in the reactor were adjusted to obtain a desired polymer molecular weight. Enzyme solution and H_2O_2 were added in pulses to optimize the utilization of the enzyme. The supernatant, containing unreacted monomer, hydrogen peroxide and enzyme, was recycled after isolating the polymer.

FTIR studies on polyphenols prepared by enzymatic processes indicated that the polymers lacked ether links and that the hydroxyl groups were intact. This fact allows derivatization of hydroxyl groups with a number of substituents to enhance the polymer functional properties. For example, the hydroxyl groups on poly(*p*-ethylphenol) were esterified with palmitoyl chloride and cinnamoyl chloride in DMF in the presence of stoichiometric amounts of pyridine [*5*]. The derivatized polymers exhibited lower melting points and/or enhanced UV absorption characteristics. FTIR spectra, shown in Figure 7 for palmitic groups substituted on the polymer backbone, revealed esterification of nearly all hydroxyl groups. In addition, the degree of esterification could be controlled by adjusting the stoichiometry of the reactants. The polymer may also be functionalized with biotin groups to bind a number of biomolecules to the polymer matrix. Cell growth factors, such as arginine-glycine-aspartic acid (RGD) tripeptide, may be attached to the polyphenol backbone.

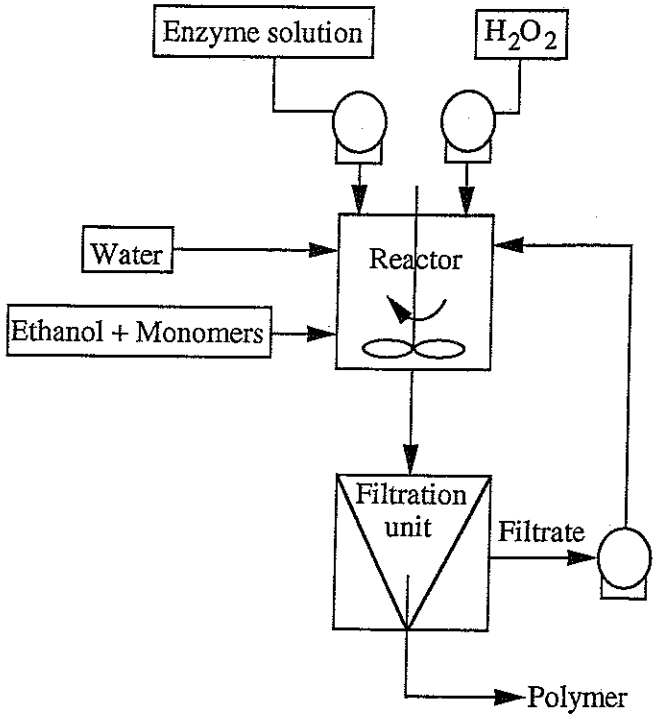


Figure 6: A schematic of the process for the production of phenolic polymers with control of polymer molecular weight and monomer conversion.

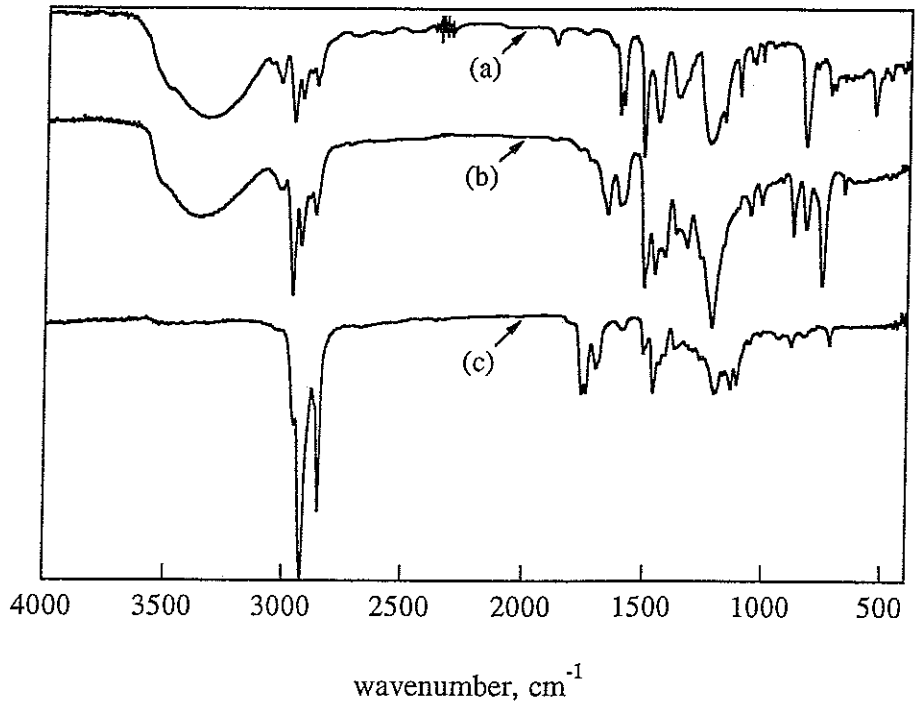


Figure 7: FTIR spectra for (a) *p*-ethylphenol, (b) poly(*p*-ethylphenol) and (c) poly(*p*-ethylphenol) derivatized at hydroxyl groups by palmitoyl chloride.

Conclusions

The molecular weight of poly(*m*-cresol) could be controlled in ethanol/water mixtures by manipulating the composition of the reaction medium. Polymer precipitation could be induced in the medium with the help of salts to curtail further chain growth. A process scheme was developed to produce polymer on a large scale while controlling the molecular weight. In addition, monomer conversion could be enhanced while recycling the reaction medium. The polymer was thermally stable, and could be derivatized at the available hydroxyl groups to tailor functional properties. In order to understand different molecular interactions in the reaction medium that are responsible for the observed dependence of polymer molecular weight on the solvent composition, enzyme activity as a function of the reaction medium composition was studied. Preliminary results indicated that the enzyme activity loss at high ethanol contents could be significantly influenced by the partitioning behavior of the monomer. Further studies on solvent-mediated alterations in enzyme structure are currently underway to gain further insight into these effects.

Acknowledgment

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